FUNGAL FLORA AND AFLATOXIN ASSOCIATED WITH COCOA, ROASTED COFFEE AND TEA POWDERS IN EGYPT

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ABSTRACT - 47 species and 2 varieties belonging to 26 genera of fungi were collected from cocoa, roasted coffee and tea powders on glucose- and cellulose-Czapek-Dox (free from sucrose) agar at 28 and 45°C. The results obtained on the two types of media were basically similar and the most common fungi (at 28°C) in the three substrates were: *Aspergillus flavus, A. fumigatus, A. niger* and *Penicillium chrysogenum*. Some fungi were prevalent only in one or two substrates on the two types of media such as *A. flavus* var. *columnaris* in cocoa and coffee, and *Mucor hiemalis* in cocoa. On the other hand some fungi were common only on cellulose agar as *A. sydowii, Cladosporium herbarum, Emericella nidulans* and *P. duclauxi* in cocoa; *P. brevicompactum, Rhizopus stolonifer* and *Stachybotrys atra* in coffee; *P. cyclopium* and *Scopulariopsis brevicaulis* in tea; and *Chaetomium globosum* in cocoa and tea. *A. fumigatus* was also the most common thermophilic (or thermotolerant) fungus in the three substrates. *Rhizomucor pusillus*, a true thermophile, was recovered rarely from cocoa and roasted coffee.

Six samples (2 and 4 samples of cocoa and tea, respectively) proved to be toxic to brine shrimp (*Artemia salina*) larvae. Thin-layer chromatographic analysis revealed that the toxic samples were contaminated by aflatoxins B_1 and B_2 (2.8-21.7μg kg). Experimental infection of the different substrates by *A. flavus* Link (CMI, 102135) proved that tea was more susceptible for aflatoxin contamination.

RÉSUMÉ - 26 genres de champignons (47 esp. + 2 var.) ont été isolés à partir de poudres de cacao, café torréfié et thé, sur milieux Czapek-glucose et -cellulose à 28 et 45°C. Les résultats obtenus sur ces deux milieux sont similaires et les champignons les plus communs, pour les 3 susbtrats et à 28°C, sont: *Aspergillus flavus, A. fumigatus, A. niger* et *Penicillium chrysogenum*. Quelques champignons n'apparaissent que sur 1 ou 2 substrats: *A. flavus* var. *columnaris* pour le café et le cacao, *Mucor hiemalis* pour le cacao. D'autres champignons sont fréquents uniquement sur milieu cellulose: *A. sydowii, Cladosporium herbarum, Emericella nidulans* et *P. duclauxi* chez le cacao; *P. brevicompactum, Rhizopus stolonifer* et *Stachybotrys atra* chez le café; *P. cyclopium* et *Scopulariopsis brevicaulis* chez le thé; *Chaetomium globosum* chez le cacao et le thé; *A. fumigatus* est le champignon thermophile (ou thermotolérant) le plus commun des 3 substrats. *Rhizomucor pusillus*, thermophile vrai, est rarement rencontré chez le café et le cacao.

6 échantillons (2 pour le cacao, 4 pour le thé) sont toxiques pour les larves d'*Artemia salina*. Les chromatographies en couche mince révèlent que ces échantillons sont contaminés par des aflatoxines B_1 et B_2. L'infection expérimentale des différents substrats par *A. flavus* montre que le thé est le plus sensible à la contamination par les aflatoxines.

KEY WORDS : Cocoa fungi, roasted coffee fungi, tea fungi, aflatoxin.

INTRODUCTION

Almost 100% of the annual world of green coffee, cocoa beans and tea are produced in developing countries. Latin America and Africa are the main producers of cocoa and coffee, whereas South of Asia is the main producer of tea in the world (FAO, 1988).

Spores of several fungi are always present in large numbers and have the ability to grow on foods, animal feeds or the raw materials used in the manufacture of these commodities. In Egypt, several investigations have been carried out on fungi contaminated seeds and grains (Moubasher et al., 1972; Abdel-Kader et al., 1979; El-Kady et al., 1982a; Abdel-Hafez et al., 1987, Abdel-Hafez, 1988; El-Maghraby & El-Maraghy, 1988; Abdel-Mallek et al., 1990), animal feed stuffs (El-Maghraby, 1989) and human foods (Abdel-Naser, 1990), but none of these studies have been conducted on cocoa, roasted coffee and tea powders commonly used in Egypt as soft drinks (with or without sugar or milk). Also, cocoa is widely used in sweet manufacture, especially chocolate.

Many filamentous fungi are able to produce a wide range of secondary metabolites. Some of these metabolites are pigments, some have antibiotic properties and others are mycotoxins. The mycotoxins are metabolites which cause illness or death on human, or his domesticated animals, following consumption of a domesticated food. The illness itself is referred to mycotoxicosis. Among these mycotoxins are the aflatoxins which are secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Their recognition as potent carcinogens in human and some animal has made them the subjects of government legislation as well as valuable tools, in the study of cancer (Moss & Smith, 1985). In Egypt, the contamination of several commodities such as seeds, grains, meat products and other food and feed stuffs have been carried out (Abdel-Hafez & El-Maghraby, 1987; Abdel-Hafez et al., 1987 a, b; El-Maghraby & El-Maghraby, 1987, 1988; Abdel-Naser, 1990).

The present investigation aimed at studying the contamination of coffee, cocoa and tea powders with filamentous fungi, as well as mycotoxins. Also, the production of aflatoxins B_1 and B_2 on the above three substrates by *Aspergillus flavus* (CMI 102135) were also studied.

MATERIALS AND METHODS

Twenty samples of each of cocoa, roasted coffee and tea powders (about 100-150g each) were collected from different markets from five Governorates in Upper Egypt namely; El-Minya, Assiut, Sohag, Qena and Aswan. Four samples of each substrate were collected from each Governorate. Each sample was placed in a sterile polyethylene bag and transferred to the Mycological laboratory and kept in a cool place (3-5°C) till fungal and mycotoxins analysis.

Determination of moisture content

Each substrate was dried in an oven for 24 h at 105°C, then cooled in a dessicator and re-weighed. The moisture content is expressed as percentage of the dry weight.

Determination of fungi

The dilution-plate method as described by Johnson & Curl (1972) was used for isolation of fungi. Czapek-Dox agar basal medium (g/litre: sodium nitrate 3.0; magnesium sulfate 0.5; potassium chloride 0.5; iron sulfate 0.01; di-potassium hydrogen phosphate 1.0; agar-agar 15.0; pH 7.3±0.1) in which the 3% sucrose were substituted separately with either of 1% glucose or 2% microcrystalline cellulose, were used for isolation of glucophilic and cellulose-decomposing fungi respectively. These two carbon sources, added separately in basal medium, are suitable for isolation of a wide range of fungal species from the three tested substrates. Streptomycin (20 U/ml) and rose bengal (30 ppm) were added to the medium as bacteriostatic agents. Fifteen plates (5 plates for each type of medium and the other 5 plates for thermophilic fungi) were used for each sample. Plates were incubated at 28°C (mesophilic fungi) or 45°C (for thermophilic and thermotolerant fungi) for 1-2 weeks and the developing fungi were identified, counted and the numbers were calculated per g of each substrate.

The following references were used for the identification of fungi (purely morphologically; based on macro- and microscopic characteristics): Raper & Fennell (1965), Ellis (1971), Booth (1971), Pitt (1979), Domsch et al. (1980), Ramirez (1982), Sivanesan (1984).

Mycotoxins analysis

1- Extraction procedure

25 g of each sample of coffee, cocoa and tea powders were transferred to 250ml Erlenmeyer flask containing 150ml of 80% acetone-water and placed on rotary shaker for 24h. The solvent was decanted off and re-extraction by 150ml of acetone. The acetone extracts were combined and filtered through Whatman n° 1 filter paper. The aqueous acetone solution was reduced in volume by flash evaporation and the filtrate washed twice with 50ml n-hexane in a 250ml separatory funnels, and the n-hexane layer was discarded. The resulting solution was extracted with chloroform (three time, 50ml each). The extracts were combined and traces of water were removed with anhydrous $Na_2 SO_4$. The chloroform extract was concentrated in vacuum and the dry material was transferred to 1- dram vials with small amounts of chloroform, and the solution was evaporated to neat dryness under a stream of nitrogen.

2- Clean up of the crude extracts

The dry material was suspended in 50ml chloroform and applied to silica gel column (200 mesh, Merck) (A.O.A.C., 1980). The column was washed with 150ml n-hexane, followed by 150ml ether, and aflatoxins were eluted with 150ml of 3% methanol-chloroform, which was then concentrated under a stream of nitrogen.

3- Chemical detection of mycotoxins

For qualitative detection of mycotoxins, thin layer chromatography technique was used using precoated silica gel 60 plates (E. Merck, Germany). Aflatoxins B_1, B_2, G_1 & G_2; ochratoxins A & B; sterigmatocystin, T-2 toxin and diacetoxyscirpenol were applied as standard references. All of mycotoxin standards used through this study were kindly provided by Prof. Dr. G.A. Bean, University of Maryland, U.S.A. The developing solvent system was methanol-chloroform (V V, 3:97) and the developed plates were viewed under short wavelength UV (252nm) light (A.O.A.C., 1980). For quantitative determination of aflatox-

ins, the fluorescent zones, including standards, were removed from the plates and the aflatoxins were disolved in chloroform. The concentrations were determined spectrophotometrically (Cecil model 703 spectrophotometer) using the molecular coefficient of 21,800 at 362nm as reported by Asao et al. (1963).

Presence of ochratoxins was confirmed by formation of the fluorescent methyl esters according to the method described by Nesheim et al. (1973). Reaction with trifluoroacetic anhydried had been also applied for derivatization of ochratoxin A (Broce, 1970).

Presence of sterigmatocystin was confirmed according to the method of Josefsson & Moller (1977).

Diacetoxyscirpenol and T-2 toxins were confirmed according to the method described by Takitani et al. (1979).

Substrates and inoculation with *Aspergillus flavus*

50g of each of moistened (40% H_2O) autoclaved substrate (cocoa, roasted coffee or tea) in 500ml Erlenmeyer flask were inoculated with 5ml spore suspension (approximately $7x10^{10}$ conidia) of *Aspergillus flavus* (CMI, 102135). The cultures were incubated at 28° ± 1°C for 2 weeks and then at 10° ± 1°C for another 2 weeks. 25g of the samples were transferred to 250ml Erlenmeyer flask containing 100ml of chloroform and placed on rotary shaker for 24h. The chloroform extract was filtered, concentrated, transferred to 1-dram vial, and the solution was evaporated under a stream of nitrogen. The dry material was cleaned up as previously mentioned.

Brine shrimp test

For mycotoxins-bioassay, brine shrimp (*Artemia salina* L.) larvae were used according to the method described by Korpinen (1974). 20μg of the previous extracts were added to 2ml sea water containing 60-100 larvae. After 24h, the percentage of mortality was determined.

RESULTS AND DISCUSSION

The moisture content of cocoa, roasted coffee and tea powder samples was considerably low and ranged between 2.8-3.9%, 2.1-4.9% and 5.3-8.1%, respectively.

Glucophilic fungi (species growing on Czapek-Dox medium with glucose at 28°C)

The total count of filamentous glucophilic fungi in samples tested widely fluctuated between 120-1050, 110-980 and 160-1280 colonies g of cocoa, roasted coffee and tea powders, respectively. It is worthy to mention that samples with high values of moisture contents coincided with high numbers of fungi and vice versa.

Forty-four species and one variety belonging to 25 genera were collected from cocoa (11 genera, 23 species + 1 var.), coffee (16 genera, 26 species + 1 var.) and tea powders (11 genera, 24 species + 1 var.) on glucose-Czapek-Dox agar (free from sucrose) at 28°C (Tables 1-2). All of these fungi were firstly isolated from the above three substrates in Egypt, but almost the majority of them were found previously from seeds and grains by several researchers.

Genera	Glucose-Cz.						Cellulose-Cz.					
	Cocoa		Coffee		Tea		Cocoa		Coffee		Tea	
	%C	%F	%C	%F	%C	%F	%C	%F	%C	%F	%C	%F
Acremonium (1)	-	-	-	-	0.7	10	-	-	0.5	10	-	-
Alternaria (1)	-	-	-	-	0.3	10	-	-	0.5	5	-	-
Aspergillus (11+1 variety)	84.1	100	74.4	100	74.7	100	79.2	100	71.8	100	75.7	75
Botryotrichum (1)	-	-	0.3	5	-	-	-	-	-	-	-	-
Chaetomium (1)	-	-	-	-	0.8	10	0.7	15	0.9	10	3.7	20
Cladosporium (3)	-	-	3.3	40	2.1	35	0.8	15	2.0	20	0.2	5
Cochliobolus (1)	-	-	0.1	5	-	-	-	-	-	-	-	-
Cunninghamella (1)	0.5	5	-	-	-	-	-	-	-	-	-	-
Emericella (1)	0.3	10	-	-	-	-	0.9	15	0.7	10	0.1	5
Eurotium (1)	0.1	5	-	-	-	-	-	-	-	-	-	-
Fennellia (1)	-	-	0.3	5	-	-	-	-	-	-	-	-
Fusarium (1)	0.4	5	0.3	5	-	-	-	-	-	-	-	-
Gibberella (1)	5.6	5	-	-	-	-	6.4	5	-	-	-	-
Geotrichum (1)	-	-	2.4	20	-	-	-	-	-	-	-	-
Mucor (2)	1.7	30	0.5	5	1.1	15	2.4	45	1.9	20	-	-
Myrothecium (1)	-	-	0.6	25	-	-	-	-	0.7	10	-	-
Nectria (1)	-	-	0.3	5	-	-	-	-	0.3	5	-	-
Paecilomyces (1)	0.2	10	0.5	15	3.9	40	1.5	25	1.3	15	3.7	20
Penicillium (9)	5.9	60	12.7	70	14.4	55	7.0	50	13.8	55	14.0	40
Rhizopus (1)	0.2	5	0.1	10	0.3	10	-	-	2.6	20	0.1	10
Scopulariopsis (1)	-	-	0.7	5	1.0	15	0.6	10	0.6	5	2.5	25
Stachybotrys (1)	-	-	0.1	5	-	-	-	-	2.2	20	-	-
Syncephalastrum (1)	1.0	20	-	-	-	-	-	-	-	-	-	-
Trichoderma (1)	-	-	0.1	5	-	-	-	-	-	-	-	-
Viocladium (1)	-	-	-	-	0.7	10	-	-	-	-	-	-
Mycelia sterilia	-	-	2.4	25	-	-	0.5	10	0.2	5	-	-
% Total count	100		100		100		100		100		100	
Number of genera = 25	11		16		11		9		14		8	
Number of species=46+1 var.	23+1 var.		26+1 var.		24		18+1 var.		23+1 var.		14	

Figures between parenthesis refer to the number of species.

Table 1. - Percentage counts (%C, calculated per total fungi) and percentage frequency of occurrence (%F, calculated per 20 cases) of various fungal genera recovered from cocoa, coffee and tea powders on glucose- and cellulose-Czapek-Dox (free from sucrose) agar at 28°C.

Tableau 1. - Fréquences d'isolement des différents genres de champignons obtenus à partir des poudres de cacao, café et thé sur milieux Czapek-glucose et -cellulose à 28°C.

Aspergillus was the most prevalent genus and was encountered from all samples comprising 84.1, 74.4 and 74.7% of total fungi in cocoa, coffee and tea, respectively. It was represented by 9 species and 1 variety (9 + 1, 6 + 1 var. and 7 species in cocoa, coffee and tea, respectively) of which *A. flavus*, *A. fumigatus* and *A. niger* were the most common in the three substrates. They emerged in

Table 2. - Total counts (TC, calculated per g dry substrate), number of cases of isolation (NCI, out of 20) and occurrence remarks (OR) of various fungal genera and species recovered from cocoa, coffee and tea powders on glucose- and cellulose-Czapek-Dox (free from sucrose) agar at 28°C.

Tableau 2. - Genres et espèces de champignons isolés à partir de poudres de thé, café et cacao, sur milieux Czapek-glucose et -cellulose à 28°C.

Genera and species	Glucose-Cz.						Cellulose-Cz.					
	Cocoa		Coffee		Tea		Cocoa		Coffee		Tea	
	TC	NCI & OR	TC	NCI & OR	TC	NCI & OR	TC	NCI & OR	TC	NCI & OR	TC	NCI & OR
Acremonium strictum W. Gams	-	-	-	-	80	2 R	-	-	30	2 R	-	-
Alternaria alternata (Fries) Keissler	-	-	-	-	40	2 R	-	-	30	1 R	-	-
Aspergillus (total count)	8970	20 H	6080	20 H	9140	20 H	6210	20 H	4920	20 H	6590	15 H
A. candidus Link	-	-	-	-	-	-	-	-	20	1 R	-	-
A. flavus Link	2260	17 H	2210	19 H	2020	13 H	1870	15 H	1180	12 H	1620	11 H
A. flavus var. *columnaris* Raper & Fennell	1710	14 H	1630	11 H	-	-	1080	18 H	1940	15 H	-	-
A. fumigatus Fresenius	3110	19 H	580	9 M	2050	14 H	2450	19 H	340	6 M	1500	13 H
A. niger Van Tieghem	1570	15 H	1390	18 H	4540	19 H	650	13 H	1320	17 H	3260	16 H
A. niveus Blochwitz	30	2 R	-	-	-	-	20	1 R	-	-	-	-
A. ochraceus Wilhelm	-	-	-	-	-	-	10	1 R	-	-	-	-
A. parasiticus Speare	120	2 R	-	-	280	3 L	-	-	-	-	-	-
A. sydowii (Bain. & Sart.) Thom & Church	110	3 L	140	4 L	200	3 L	110	4 L	-	-	-	-
A. versicolor (Vuill.) Tirab.	20	2 R	-	-	-	-	-	-	-	-	10	1 R
A. tamarii Kita	-	-	20	1 R	20	1 R	20	2 R	-	-	-	-
A. terreus Thom	40	4 L	110	3 L	30	1 R	-	-	120	2 R	-	-
Botryotrichum atrogriseum Van Beyma	-	-	20	1 R	-	-	-	-	-	-	-	-
Chaetomium globosum Kunze	-	-	-	-	100	2 R	50	3 L	60	2 R	320	4 L
Cladosporium (total count)	-	-	270	8 M	260	7 M	60	3 R	140	4 R	20	1 R
C. cladosporioides (Fres.) de Vries	-	-	120	3 L	140	4 L	20	2 R	80	3 R	20	1 R
C. herbarum (Pers.) Link	-	-	140	6 M	100	3 L	40	3 L	60	2 R	-	-
C. microcarpum Preuss	-	-	10	1 R	20	1 R	-	-	-	-	-	-
Cochliobolus spicifer Nelson	-	-	10	1 R	-	-	-	-	-	-	-	-
Cunninghamella echinulata Thaxter	50	1 R	-	-	-	-	-	-	-	-	-	-
Emericella nidulans (Eidam) Vuillemin	30	2 R	-	-	-	-	70	3 L	50	2 R	10	1 R
Eurotium chevalieri Mangin	10	1 R	-	-	-	-	-	-	-	-	-	-
Fennellia flavipes Wiley & Simmons	-	-	20	1 R	-	-	-	-	-	-	-	-
Fusarium oxysporum Schlecht	40	1 R	20	1 R	-	-	-	-	-	-	-	-

Genera and species	Glucose-Cz.						Cellulose-Cz.					
	Cocoa		Coffee		Tea		Cocoa		Coffee		Tea	
	TC	NCI & OR	TC	NCI & OR	TC	NCI & OR	TC	NCI & OR	TC	NCI & OR	TC	NCI & OR
Gibberella fujikuroi (Sawada) Ito	600	1 R	-	-	-	-	500	1 R	-	-	-	-
Geotrichum candidum Link	-	-	200	4 L	-	-	-	-	-	-	-	-
Mucor (total count)	180	6 M	40	1 R	140	3 L	190	9 M	130	4 L	-	-
M. circinelloides Van Tieghem	120	3 L	-	-	140	3 L	-	-	130	4 L	-	-
M. hiemalis Wehmer	60	4 L	40	1 R	-	-	190	9 M	-	-	-	-
Myrothecium verrucaria (Alb. & Sch.) Dit.	-	-	50	5 L	-	-	-	-	50	2 R	-	-
Nectria haematococca Berkeley & Brown	-	-	20	1 R	-	-	-	-	20	1 R	-	-
Paecilomyces variotii Bainier	20	2 R	40	3 L	480	8 M	120	5 L	90	3 L	320	4 L
Penicillium (total count)	630	12 H	1040	14 H	1760	11 H	550	10 M	950	11 H	1220	8 M
P. albidum Sopp	-	-	-	-	60	1 R	-	-	-	-	-	-
P. brevi-compactum Dierckx	60	2 R	80	3 L	-	-	-	-	160	5 L	-	-
P. chrysogenum Thom	420	11 H	310	8 M	1100	9 M	210	8 M	150	4 L	720	5 L
P. citrinum Thom	60	1 R	60	2 R	80	3 L	-	-	-	-	20	1 R
P. corylophilum Dierckx	10	1 R	-	-	-	-	-	-	-	-	-	-
P. cyclopium Westling	-	-	-	-	340	5 L	-	-	30	1 R	360	3 L
P. duclauxii Delacroix	-	-	-	-	-	-	230	5 L	70	2 R	-	-
P. funiculosum Thom	80	2 R	590	4 L	160	3 L	110	4 L	540	6 M	120	2 R
P. jensenii Zaleski	-	-	-	-	20	1 R	-	-	-	-	-	-
Rhizopus stolonifer (Ehrenb.) Lindt	20	1 R	80	2 R	30	2 R	-	-	180	4 L	10	1 R
Scopulariopsis brevicaulis (Sacc.) Bainier	-	-	60	1 R	120	3 L	60	2 R	40	1 R	220	5 L
Stachybotrys atra Corda	-	-	10	1 R	-	-	-	-	150	4 L	-	-
Syncephalastrum racemosum (Cohn) Schroeter	110	4 L	-	-	-	-	-	-	-	-	-	-
Trichoderma viride Pers.	-	-	10	1 R	-	-	-	-	-	-	-	-
Ulocladium botrytis Preuss	-	-	-	-	80	2 R	-	-	-	-	-	-
Mycelia sterilia (White & dark colour)	-	-	200	5 L	-	-	40	2 R	10	1 R	-	-
Gross total count	10660		8170		12230		7840		6850		8710	
Number of genera = 25	11		16		11		9		14		8	
Number of species = 46+1 var.	23+1 var.		26+1 var.		24		18+1 var.		23+1 var.		14	

Occurrence remakrs (OR): H= high occurrence (between 11-20 cases; out of 20), M= moderate occurrence (between 6-10 cases), L= low occurrence (between 3-5 cases), R= rare occurrence (1 or 2 cases).

45-95% of the samples contributing 9.54-49.67% of total *Aspergillus* and 7.1-37.1% of total fungi. *A. flavus* var. *columnaris* was recovered only, in high incidence, from cocoa and coffee. It occurred in 70 and 55% of the samples matching 19.06 and 26.8% of total *Aspergillus* and 16.04 and 19.95% of total fungi, respectively. Abdel-Kader & Al-Hubaishi (1985) isolated 12 species of *Aspergillus* from 20 samples of coffee fruits collected from Yemen Arab Republic and the most common species were *A. flavus, A. niger, A. ustus* and *A. terreus*. The above species were also common on animal feed stuffs, human food and various types of seeds and grains in Egypt (Moubasher et al., 1972; El-Kady et al., 1982a,b; Abdel-Hafez et al., 1987a; Abdel-Hafez, 1988; Abdel-Naser, 1990) or in many parts of the world (Salcado & De Carvalho, 1980; Supriaman & Palmer, 1981. Abdel-Hafez, 1984). *A. sydowii* and *A. terreus* in three substrates; *A. niveus* and *A. versicolor* in cocoa; *A. parasiticus* in cocoa and tea; and *A. tamarii* in coffee and tea were isolated in low or rare frequency of occurrence. The above *Aspergillus* species were infrequently encountered from various substrates in different places of the world (Domsch et al., 1980).

Penicillium ranked second in the number of cases of isolation and was recovered from 60, 70 and 55% of the samples constituting 5.9, 12.7 and 14.4% of total fungi in cocoa, coffee and tea, respectively. Of the genus 8 species were collected of which *P. chrysogenum* was the most common in the three substrates. It occurred in 55, 40 and 45% of the samples matching 66.6, 29.8 and 62.5% of total *Penicillium* and 3.94, 3.79 and 8.99% of total fungi, respectively. *P. citrinum* and *P. funiculosum* in the three substrates; *P. brevi-compactum* in cocoa and coffee; *P. corylophilum* in cocoa; and *P. albidum, P. cyclopium* and *P. jensenii* in tea were encountered in low or rare incidence. Levi & Barker (1968) isolated several members of *Penicillium* from green coffee beans. Abdel-Kader & Al-Hubashi (1985) collected 11 species of *Penicillium* from coffee fruits in Yemen and the most common were *P. chrysogenum, P. funiculosum* and *P. notatum*. Also the previous species have been found to contaminate various types of seeds and grains in Egypt (Moubasher et al., 1972; El-Kady et al., 1982a; Abdel-Hafez et al., 1987; Abdel-Mallek et al., 1990; El-Maghraby & El-Maraghy, 1987), as well as on various substrates all over the world (Domsch et al., 1980).

Cladosporium (represented by *C. cladosporioides, C. herbarum* and *C. macrocarpum*) in coffee and tea; *Mucor (M. circinelloides* and *M. hiemalis*) in cocoa; and *Paecilomyces (P. variotii)* in tea were recovered in moderate frequency of occurrence, but in the other substrates were completely missed or encountered in low or rare incidence (Table 2). *C. cladosporioides, C. herbarum, C. macrocarpum* and *M. hiemalis* had been found, but with various numbers and incidence, on coffee fruits from Yemen (Abdel-Kader & El-Hubaishi, 1985).

Acremonium (A. strictum), Alternaria (A. alternata), Botryotrichum (B. atrogriseum), Chaetomium (C. globosum), Cochliobolus (C. spicifer), Cunninghamella (C. echinulata), Emericella (E. nidulans), Eurotium (E. chevalieri), Fennellia (F. flavipes), Fusarium (F. oxypsorum), Gibberella (G. fujikuroi), Geotrichum (G. candidum), Myrothecium (M. verrucaria), Nectria (N. haematococca), Rhizopus (R. stolonifer), Scopulariopsis (S. brevicaulis), Stachybotrys (S. atra), Syncephalastrum (S. racemosum), Trichoderma (T. viride), Ulocladium (U. botrytis) and mycelia sterilia were infrequently encountered from one or more substrates (Table 2). Most of these fungi were associated with various types of seeds and grains, food and feed stuffs and other substrates in Egypt and in many parts of the world as reported by several investigators.

Cellulose-decomposing fungi (species growing on Czapek-Dox medium with cellulose at 28°C)

Thirty-one species and 1 variety belonging to 15 genera were collected from cocoa (9 genera, 18 species + 1 var.), coffee (14 genera, 23 species + 1 var.) and tea powders (8 genera, 14 species) on plates of cellulose-Czapek-Dox (free from sucrose) agar at 28°C (Tables 1-2). The total count of cellulose-decomposing fungi ranged between 100-860, 80-660 and 120-740 colonies/g dry weight of each of cocoa, roasted coffee and tea respectively. The results obtained in plates of cellulose agar were basically similar to those on glucose agar and the most common fungi in the three substrates were: *Aspergillus flavus, A. fumigatus, A. niger, Paecilomyces variotii* and *Penicillium chrysogenum.* Some fungi were prevalent in one ot two substrates such as *A. flavus* var. *columnaris* and *P. funiculosum* in cocoa and coffee; *A. sydowii, Cladosporium herbarum, Emericella nidulans, Mucor hiemalis* and *P. duclauxii* in cocoa; *M. circinelloides, P. brevi-compactum, Rhizopus stolonifer* and *Stachybotrys atra* in coffee; *P. cyclopium, Scopulariopsis brevicaulis* in tea; and *Chaetomium globosum* in cocoa and tea. Al-Hubaishi & Abdel-Kader (1985) found that the most prevalent fungi associated with coffee fruits of Yemen on plates of cellulose agar were: *Alternaria alternata, Aspergillus flavus, A. niger, A. ochraceus, Chaetomium globosum, Cladosporium herbarum, Fusarium oxysporum, Penicillium notatum* and *Phoma humicola.* Several of the above fungi were encountered, but with different numbers and frequency of occurrence, from Egyptian seeds and grains on cellulose agar plates (Abdel-Hafez & Abdel-Kader, 1980; Mazen et al., 1984; Abdel-Hafez et al., 1987a; Abdel-Hafez, 1988). Also, all fungal species recovered on plates of cellulose agar were reported to be cellulose-decomposing but with different ability as reported by several researchers. The remaining species were less frequently encountered (Table 2).

Thermophilic and thermotolerant fungi (species growing on Czapek-Dox medium with glucose at 45°C).

Seven species and 1 variety belonging to 4 genera were collected from cocoa (3 genera, 6 species), coffee (4 genera, 7 species) and tea powders (3 genera, 5 species + 1 var.) on plates of glucose-Czapek-Dox (free from sucrose) agar at 45°C (Table 3). *Aspergillus fumigatus* was the most common species in the three substrates. It occurred in 100, 60 and 80% of the samples matching 93.88, 61,7 and 94.8% of total *Aspergillus* and 89.03, 56.8 and 89.57% of total fungi in cocoa, roasted coffee and tea powders, respectively. *A. fumigatus* has long bean known as thermophilic fungus. But Cooney & Emerson (1964) consider it as thermotolerant as it has a maximum near to 50°C, but a minimum well below 20°C. This species was also the most common thermiophilic (or thermotolerant) fungus found on peanuts (Moubasher et al., 1979), anise, caraway, coriander, cumin and funnel seeds in Egypt (Abdel-Hafez et al., 1987b), as well as on freshly harvested rice seeds from Malaysia (Kuthubutheen, 1979). Moubasher et al., (1982) found that *A. fumigatus* was active colonizer of wheat and broad-bean straws. Sellars et al. (1970) mentioned that *A. fumigatus* could degrade barley husk and produce 1,4 β-glucanase and β-glucosidase. *A. flavus, A. niger, Emericella nidulans, E. nidulans* var. *latus, Paecilomyces variotii, Rhizomucor pusillus* and sterile mycelium were infrequently encountered from one or more substrates. These fungi were also encountered, but with different numbers and incidences, from some Egyptian seeds on plates of glucose-Czapek's medium at 45°C (Moubasher et al.,, 1979; Abdel-Hafez et al., 1987b). Thermophilic and or thermotolerant fungi were able to cause serious deterioration of palm kernels (Oso, 1979) and peanuts seeds (Moubasher et al., 1979) during storage.

Genera and species	Cocoa		Coffee		Tea	
	TC	NCI & OR	TC	NCI & OR	TC	NCI & OR
Aspergillus (total count)	735	20 H	405	18 H	770	19 H
A. flavus Link	25	3 L	110	5 L	15	2 R
A. fumigatus Fresenius	690	20 H	250	12 H	730	16 H
A. niger Van Tieghem	10	2 R	10	1 R	25	2 R
A. terreus Thom	10	2 R	35	4 L	-	-
Emericella (total count)	20	3 L	10	2 R	5	1 R
E. nidulans (Eidam) Vuillemin	20	3 L	10	2 R	-	-
E. nidulans var. *latus* Thom & Raper	-	-	-	-	5	1 R
Paecilomyces variotii Bainier	-	-	15	1 R	25	2 R
Rhizomucor pusillus (Lindt) Schipper	15	2 R	5	1 R	-	-
Sterile mycelium	5	1 R	5	2 R	15	3 L
Gross total count	775		440		815	
Number of genera = 4	3		4		3	
Number of species = 7+1 var.	6		7		5+1 var.	

Occurrence remarks (OR): H= high occurrence (between 11-20 cases; out of 20), M= moderate occurrence (between 6-10 cases), L= low occurrence (between 3-5 cases), R= rare occurrence (1 or 2 cases).

Table 3. - Total counts (TC, calculated per g dry substrate), number of cases of isolation (NCI, out of 20) and occurrence remarks (OR) of various fungal genera and species recovered from cocoa, coffee and tea powders on glucose-Czapek-Dox (free from sucrose) agar at 45°C.

Tableau 3. - Genres et espèces de champignons isolés à partir de poudres de cacao, café et thé, sur milieu Czapek-glucose à 45°C.

Natural occurrence of aflatoxin

The toxicity test using brine shrimp larvae revealed that the extracts of 2 and 4 samples of cocoa and tea, respectively, were toxic to the test organism. Thin layer chromatographic (TLC) analysis proved that aflatoxins B_1 & B_2 were present in different cocoa and tea substrates (12.6-21.7 and 2.8-18.4 μg/kg in each of cocoa and tea, respectively) (Table 4). These six samples were heavily contamined with one or two members of *Aspergillus flavus* group (*A. flavus*, *A. flavus* var. *columnaris* or *A. parasiticus*) (Table 4). Roasted coffee samples proved to be free from aflatoxins B_1 & B_2 or other toxins. In this respect, aflatoxin and sterigmatocystin have been previously reported to contaminate green

Substrate number	Moisture content (%)	Brine shrimp* test (% of dead larvae)	Aflatoxin identified (µg/kg)	Aflatoxins producing fungi
Cocoa				
7	3.9	A	B_1 & B_2 (21.7 µg)	*A. flavus, A. parasiticus*
10	3.7	■	B_1 ■ B_2 (12.6 µg)	*A. flavus, A. flavus* var. *columnaris*
Tea				
2	5.8	C	B_1 & B_2 (3.6 µg)	*A. flavus*
3	7.3	C	B_1 & B_2 (4.2 µg)	*A. flavus, A. parasiticus*
7	8.1	B	B_1 & B_2 (18.4 µg)	*A. flavus, A. parasiticus*
20	6.2	C	B_1 & B_2 (2.8 µg)	*A. flavus*

* Brine shrimp test: A= high toxicity, more than 75% mortality of brine shrimp larvae; B= moderate toxicity, between 50-75% mortality of brine shrimp larvae; C= low toxicity, between 25-49 mortality of brine shrimp larvae.

Table 4. - Sample number, substrate type, moisture content (%), biological assay, naturally occurring of aflatoxins identified and common aflatoxin -producing fungi of the toxic samples

Tableau 4 - Test et composition en aflatoxines des échantillons toxiques.

Substrate (25g)	Brine shrimp* test	Aflatoxins production** (µg/kg dry substrate)
Cocoa	A	30
Coffee	C	1.4
Tea	A	72

* Brine shrimp test: A= high toxicity, more than 75% mortality of brine shrimp larvae; B= moderate toxicity, between 50-75 mortality of brine shrimp larvae; C= low toxicity, between 25-49 mortality of brine shrimp larvae.

** Aflatoxins production: Average of two determination of UV spectrophotometer, where the individual analysis agreed to within ± 10%.

Table 5. - Brine shrimp test and production of aflatoxins on moistened cocoa, roasted coffee and tea powders in relation to their requirements by *Aspergillus flavus* (CMI, 102135).

Tableau 5. - Infection expérimentale de différents substrats par *Aspergillus flavus*, test et production d'aflatoxines.

coffee beans at ■ level of 20-400µg/kg (Levi & Barker, 1968; Schroedar & Storey, 1976; FAO, 1979). Also aflatoxins B_1 & B_2 were the only mycotoxins which have been previously recorded as a contaminant of cocoa beans at concentration of 4.87µg/kg and also in cocoa products (chocolates) in USA at a level of 10µg/kg (FAO, 1979). In Egypt, aflatoxins have been identified as natural contaminant of some seeds and grains (El-Khadem et al., 1983; Youssef,

1986; El-Maghraby & El-Maraghy, 1987; Sabah Saber, 1987; El-Maghraby, 1989).

Biological assay, TLC analysis and U.V. spectrophotometer proved clearly that tea was more susceptible for contamination by aflatoxins B_1 & B_2 than cocoa (72 and 30μg kg dry tea and cocoa, respectively) (Table 5). Coffee is weakly susceptible for production aflatoxins (1.4μg kg coffee). Aflatoxins generally refer to a group of toxic crystalline, highly fluorescent bis-furanocoumarin metabolites produced by *Aspergillus flavus* and *A. parasiticus* (Edds, 1979). Aflatoxin B_1 causes chromosomal aberration and DNA breakage in plant and animal cell. It has been demonstrated to cause mutations in several bacterial test systems (Edds, 1979; Moss & Smith, 1985). El-Zawahri et al. (1977) demonstrated that aflatoxin B_1 is a strong chromosome damaging agent and the treated cells showed a high rate of breaks and interchanges.

In conclusion, mycological analysis of cocoa, coffee and tea powders reveal that these substrates were contaminated with several glucophilic and cellulose-decomposing fungi, especially members of *Aspergillus* and *Penicillium*, but there is no specific fungal characteristics any of these substrates. Also, *A. flavus* was encountered in high frequency of occurrence in the three substrates on glucose- and cellulose-Czapek's agar, where this fungus is well known glucophile, cellulose-decomposer and aflatoxins-producing fungus and was found in large numbers on the contaminated six samples of cocoa an tea powders by aflatoxins B_1 and B_2. Hence, precautions must be adopted during handling, transport, storage and processing to avoid contamination and serious deterioration of the three substrates by filamentous fungi, since several of these fungi could produce mycotoxins which are harmful to human health. Also some samples of cocoa and tea proved to be contaminated with aflatoxins B_1 & B_2 and these two substrates were more susceptible for production of aflatoxins than roasted coffee.

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